

Antioxidant and antiproliferative activity of *Stachys glutinosa* L. ethanol extract

Questa è la versione Pre print del seguente articolo:

*Original*

Antioxidant and antiproliferative activity of *Stachys glutinosa* L. ethanol extract / Lidia, Leporini; Luigi, Menghini; Foddai, Marzia; Petretto, Giacomo Luigi; Chessa, Mario; Bruno, Tirillini; Pintore, Giorgio Antonio Mario. - In: NATURAL PRODUCT RESEARCH. - ISSN 1478-6419. - 29:10(2015), pp. 899-907. [10.1080/14786419.2014.955490]

*Availability:*

This version is available at: 11388/61139 since: 2022-06-07T10:54:06Z

*Publisher:*

*Published*

DOI:10.1080/14786419.2014.955490

*Terms of use:*

Chiunque può accedere liberamente al full text dei lavori resi disponibili come "Open Access".

*Publisher copyright*

note finali coverpage

(Article begins on next page)

# ANTIOXIDANT, ANTIPROLIFERATIVE, AND ANTIMICROBIAL ACTIVITY OF ETHANOL EXTRACT OF *STACHYS GLUTINOSA* L.

Lidia Leporini,<sup>a</sup> Nadia Scanu,<sup>a</sup> Luigi Menghini,<sup>a</sup> Giacomo Luigi Petretto,<sup>b</sup> Bruno Tirillini,<sup>c</sup> Andrea Piana,<sup>d</sup> Maria Dolores Masia<sup>d</sup> and Giorgio Pintore<sup>b</sup>

a,Department of Drug Science University G. d'Annunzio, Chieti, Italy

b,Department of Chemistry and Pharmacy, University of Sassari, Italy

c,Department of Human, Environmental and Natural Sciences, University of Urbino, Urbino (PG), Italy

d,Department of Scienze Biomediche University of Sassari, Via P. Manzella, 4, Italy

e-mail for correspondence: [pintore@uniss.it](mailto:pintore@uniss.it)

## ABSTRACT

Ethanol extracts of *Stachys glutinosa* L. (Lamiaceae) were investigated for the antioxidative properties, as well antiproliferative action on tumor cells and antimicrobial activities. The antioxidant activities were investigated by 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging,  $\beta$ -carotene/linoleic acid assay, scavenging of hydrogen peroxide (HRPO test), superoxide anion scavenging (S.A.S. test), and hypochlorous acid scavenging (taurine test). The antioxidant activity was reported as IC<sub>50</sub> and reveals antioxidant effects. Antiproliferative effects were measured *in vitro* on three cell lines: HepG2 (human hepatocarcinoma), MCF7 (breast adenocarcinoma) and C2C12 (mouse myoblast) cell lines by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Extract induces variation of cell viability on all cell lines tested. At 200  $\mu$ g/ml, the effects on cell viability result -23, -27, -37%, respectively for C2C12, MCF7 and HepG2). Ethanol extract exhibits also antibacterial activity against *E. coli* and *S. pyogenes*.

## Introduction

It is well known that oxygen is essential for many living organisms. In contrast metabolites of oxygen (reactive oxygen species ROS), like free radicals are potentially harmful and can give rise to so called oxidative stress [A]. Organisms defend themselves against ROS by different antioxidant systems, mainly identified as enzymes, vitamins, trace elements and natural products that can be introduced as food, by diet, or as

nutraceuticals. [B, C] A large literature shows that wild flora still represent a promising source of plant extracts and phytochemicals of antioxidant activity [D, E, F].

After evidence of potential dangerous effects for health from synthetic antioxidant, such as of as tert-butylhydroquinone and tert-butyl-4-hydroxyanisole, nowadays a renewed interest is observed for medicinal plant with antioxidant activity as potential food additives [G]

The antioxidant activity of plant extracts is mainly due to substances known as phenolic compounds. The antioxidant activity is often directly related to the presence of this biomolecules in plants and extracts. [H]

*Stachys glutinosa* L.(Lamiaceae) is a fruticose dwarf shrub, widespread in Sardinia, Corsica and Capraia Islands [1], very common on different substrata from the sea level to higher mountain. The whole plant is covered by weak thorny stems emanating an unpleasant smell [2]. The plant is used for medicinal purposes (mainly as antispasmodic and antiseptic) in folk medicine [3]. The phytochemical composition of this specie results marginally studied. The compositions of the essential oil of *S. glutinosa* from Corsica [4, 5] and those obtained from wild plants collected in Sardinia were studied [6] The iridoidic composition of *Stachys glutinosa* L. was also examined and the presence of the known harpagide and acetyl-harpagide were showed together with that of a new di-glycosidic iridoid, the 5-alloxyloxy-aucubin. The presence of allose in *Stachys* genus seems to be a chemotaxonomical character [7]. The biological activity of this specie result unexplored, except for a antimicrobial screening that reveal activity against *Vibrio cholerae*, *Candida* strains and *Rhodotorula rubra*. In order to evaluate the potential biological activity of *S. Glutinosa*, the ethanolic extract was tested for antioxidant, antiproliferative and antimicrobic activities.

## 2. Materials and methods

### 2.1 Chemicals

All chemicals were obtained from Sigma-Aldrich Co ( St. Louis, MO) and the solvents were from Merck (Darmstad, Germany) unless otherwise indicated. All of the reagents were prepared in distilled water.

### 2.2 Plant material

*Stachys glutinosa* was collected in the flowering period (April-July 2010), from Santa Maria's Island (Sardinia). A specimen has been deposited in the Herbarium SASSA at the N° 1099 in the Department of Chemistry and Pharmacy, University of Sassari. Aerial part of plant were cut into small pieces and powdered. The plant material was extracted in ethanol by maceration. The extract was filtered, evaporated to dryness in vacuum and stored in the dark at -20°C, until use.

### 2.3 Total phenolics

A derived method of Folin-Ciocalteu, according to Singleton et al. (1965) [8] was used: 7 ml distilled H<sub>2</sub>O, 0.5 ml Folin-Ciocalteu reagent and 0.5 ml of extract were mixed. After 3 min, 2 ml of 20% Na<sub>2</sub>CO<sub>3</sub> were added and heated at 100°C during 1 min, in a water bath, comparatively to a gallic acid standard. Absorbance was measured at 685 nm after cooling in darkness and the results expressed in mg of gallic acid/100 g sample. All values were uniformly expressed referring to corresponding dry extract weight. All measures were repeated three times and averaged.

#### *2.4 Flavonoids content*

The flavonoids content was estimated by the AlCl<sub>3</sub> method: 1 ml of ethanol extract solution was added to 1 ml of 2% ethanol AlCl<sub>3</sub>· 6H<sub>2</sub>O. The absorbance was measured 10 min later at 430 nm comparatively to a rutin standard. The results were expressed in mg rutin/100 g sample. All values were uniformly expressed referring to corresponding plant dry weight. All measures were repeated three times and averaged [9].

#### *2.5 Total flavanols content*

The flavanol content was expressed in mg of (-) epicatechin:100 g sample by comparison with standard (-) epicatechin treated in the same conditions. One milliliter of ethanol extract was added to 5 ml of 0.1% (w:v) *p*-dimethylaminocinnamaldehyde in ethanol : HCl (3:1; v:v) reagent. The absorbance was measured 10 min later at 640 nm. All values were uniformly expressed referring to corresponding plant dry weight. All measures were repeated three times and averaged [10].

#### *2.6 Oligomeric proanthocyanidin content*

The proanthocyanidin content was expressed in mg of cyanidin chloride/100 g sample after heating the extracts in *n*-butanol : HCl following the method of Porter et al. (1986) [11]: 0.5 ml of ethanol extract, 6 ml of *n*-butanol : HCl (95:5; v:v) and 0.2 ml of 2% (w:v) solution of NH<sub>4</sub>Fe(SO<sub>4</sub>)<sub>2</sub>· 12H<sub>2</sub>O in 2 M HCl were mixed. The tightly capped tubes were heated during 40 min at 92°C in a water bath. After cooling, the colored solutions were measured at 550 nm. All values were uniformly expressed referring to corresponding plant dry weight. All measures were repeated three times and averaged.

#### *2.7 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay*

The antioxidant capacity assay was carried out using the improved 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS)<sup>+</sup> radical cation decolorization assay as described by Re et al. (1999) [12]. ABTS<sup>+</sup> radical cation was generated by oxidation of ABTS with potassium persulfate. ABTS was dissolved in deionized water to 7 mM concentration, and mixed with 2.45 mM potassium persulfate. The reaction mixture was left to stand at room temperature in the dark for 12–16 h before use. The ABTS<sup>+</sup>

solution was diluted with ethanol to an absorbance of  $0.700 \pm 0.020$  at 734 nm. Then variable amounts of ethanol extract were added to ABTS solution to a total volume of 1 ml and allowed to react for 5 min. Control (without sample) was used as blank. Trolox was used, as reference antioxidant substance (ranging concentration 0-15  $\mu\text{M}$ ). Appropriate solvent blanks were run in each assay. The activity was calculated as 50% inhibition concentration ( $\text{IC}_{50}$ ). All determinations were carried out in triplicate and averaged.

### *2.8 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity*

The antioxidant activity was determined also by the DPPH radical-scavenging method according to Peterson and Hahn (2002) [13]. 100  $\mu\text{l}$  of ethanol extract of sample at different concentration was mixed with 900  $\mu\text{l}$  100 mM Tris-HCl buffer, pH 7.4, and then added to 1 ml 0.5 mM DPPH in ethanol (250  $\mu\text{M}$  in the reaction mixture). The control sample was prepared using ethanol. Trolox was employed as a standard antioxidant to compare the radical-scavenging activities. Absorbances of the mixtures were measured at 517 nm. The activity was calculated as 50% inhibition concentration ( $\text{IC}_{50}$ ). All determinations were carried out in triplicate and averaged.

### *2.9 $\beta$ -Carotene/linoleic acid assay*

In this assay, antioxidant capacity was determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation. A stock solution of  $\beta$ -carotene/linoleic acid mixture was prepared as follows: 0.5 mg  $\beta$ -carotene was dissolved in 1 ml of chloroform, then 25  $\mu\text{l}$  linoleic acid and 200 mg Tween 40 were added. Then, 100 ml distilled water saturated with oxygen (30 min, 100 ml/min) was added with vigorous shaking; 2.5 ml of this reaction mixture were dispensed into test tubes and 10  $\mu\text{l}$  portions of the ethanol extract at different concentrations were added; the emulsion system was incubated for up to 48 h at room temperature. The same procedure was repeated with the synthetic antioxidant butylated hydroxytoluene (BHT) as positive control, and a blank. After this incubation period, absorbances of the mixtures were measured at 490 nm. The activity was calculated as 50% inhibition concentration ( $\text{IC}_{50}$ ). All determinations were carried out in triplicate and averaged [14].

### *2.10 Scavenging of hydrogen peroxide*

The  $\text{H}_2\text{O}_2$  was measured according to previously published methods [15, 16]. Briefly, 10  $\mu\text{l}$  of ethanol extract of the sample at different concentration were added to 100  $\mu\text{l}$   $\text{H}_2\text{O}_2$  in a final volume (1 ml) of PBS, pH 7.4. After 15 min at  $37^\circ\text{C}$ , 1 ml phenol red (0.2 mg/ml) containing horseradish peroxidase (17 U/ml) was added; 15 min later, 50  $\mu\text{l}$  1N NaOH was added and the amount of  $\text{H}_2\text{O}_2$  was determined by measuring the absorbance at 610 nm. Trolox was used as the positive control. The activity was

calculated as 50% inhibition concentration (IC<sub>50</sub>). All determinations were carried out in triplicate and averaged.

### 2.11 Superoxide anion scavenging ( $\bullet\text{O}_2^-$ )

The scavenging of ( $\bullet\text{O}_2^-$ ) were assayed by measuring the inhibition ability of the nitroblue tetrazolium (NBT) reduction caused by ( $\bullet\text{O}_2^-$ ) generated by the xanthine oxidase/hypoxanthine system. Reduction of NBT to formazan was measured as the change in absorbency at 560 nm versus time (3 min) either in the absence of ethanol extract of sample or with increments of sample concentrations. Briefly the reaction mixture (1 ml) contains the following reagents: phosphate buffered saline (PBS, 10 mM phosphate, NaCl 150 mM, pH 7.4); hypoxanthine (2mM); xanthine oxidase (3 units); and NBT (1.2 mM). Trolox was used as the positive control. The activity was calculated as 50% inhibition concentration (IC<sub>50</sub>). All determinations were carried out in triplicate and averaged [17, 18].

### 2.12 Hypochlorous acid scavenging (HOCl)

The amount of HOCl was measured by the chlorination of taurine. Briefly, 100  $\mu\text{l}$  sodium hypochlorite (600 mM) were added to 100  $\mu\text{l}$  taurine (150 mM) and 10  $\mu\text{l}$  ethanol extract of sample at different concentration in a final volume of 1 ml PBS, pH 7.4. Absorbance was measured at 350 nm after the addition of 10  $\mu\text{l}$  2 M potassium iodide. Trolox was used as the positive control. The activity was calculated as 50% inhibition concentration (IC<sub>50</sub>). All determinations were carried out in triplicate and averaged [19-21].

### 2.13 Cytotoxic activity

Antiproliferative effects were measured *in vitro* on three cell lines: HepG2 (human hepatocarcinoma), MCF7 (breast adenocarcinoma) and C2C12 mouse myoblast cell line by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, a limited number of human cancer cells (2500/well) were seeded onto a 96-well microplate and incubated overnight. After 24h, the original medium was removed and 200  $\mu\text{L}$  of new medium containing the sample (100 and 200  $\mu\text{g}/\text{ml}$ ) was added. After incubation for 24h and 48h the amount of living cells was assayed by the addition of 20  $\mu\text{l}$  of 5 mg/ml MTT in medium. After 3h MTT was converted, in intact mitochondria, by reductase and precipitated as blue crystals. The medium was then removed, and the precipitated crystals were dissolved in 200  $\mu\text{l}$  of dimethyl sulfoxide (DMSO), and after 30 min the amount of reduced MTT was determined at 540 nm (Varian50 MPR, Microplate reader) as already described [L]. Results are expressed as mean variation percentage of cell viability compared to control untreated groups.

### 2.14 Antimicrobial activity

Extract of *S. glutinosa* were tested against different microorganism strains from American-Type Cell Culture (ATCC) reference bacteria as well as clinical isolates: 2 Gram negative bacteria (*Pseudomonas aeruginosa* (DSM 1117), *Escherichia coli* (ATCC 13706) and 4 Gram positive bacteria (*Streptococcus agalactiae* (ISS1), *Streptococcus pneumoniae* (ISS3), *Streptococcus pyogenes* (ISS2), *Staphylococcus aureus* (ATCC 25923). Antimicrobial activity was evaluated using the agar disk diffusion method. [22] [23] Bacterial strains were cultivated on Müeller Hinton broth (Oxoid, Unipath Limited, Basingstoke, UK). Cell cultures were incubated at 37°C for 24 hours before test. The cell suspension were adjusted with sterile saline solution to obtain a turbidity comparable to that of McFarland n. 0.5 standard ( $1.5 \times 10^8$  cells/ml). Müeller-Hinton agar plates were spread with 1 ml each microbial suspension, then the excess suspension was aspirated. Petri dishes of Sabouraud dextrose agar were used for yeasts. Sterile paper disks (5.0 mm in diameter) were impregnated with 10 µl test solution at concentrations 1-0.5 mg/ml and placed on the inoculated plates. Chloramphenicol were used as positive controls (30 µg). A negative control, inoculating same volume of DMSO without extract, was also included. All the experiments were conducted in triplicate and results were expressed as mean values.

### 2.15 Statistical analysis

Analysis of variance and significances were performed by One-way ANOVA with Newman-Keuls Multiple Comparison post test, using GraphPad Prism version 5.00 (GraphPad Software, San Diego, California USA).

## 3. Results and Discussion

### 3.1 Chemical components

*S. glutinosa* dried extract contains total phenols ( $93.56 \pm 3.62$  mg/g as gallic acid equivalents), proanthocyanidins ( $21.02 \pm 1.78$  mg/g calculated as cyanidine chloridrate), total flavonoids ( $15.21 \pm 2.05$  mg/g, as rutin) and total flavonols ( $6.7 \pm 1.20$  mg/g, as (-) epicatechin), as reported in Table 1. Results were expressed as mean value of three replicates and were referred to extract dry weight. Quantitative data confirm that this specie can be an interesting source of equilibrated mix of different polyphenols, also if the total amount is not one of the highest, almost referred to the genus *Stachys*. To the best of our knowledge, no references are available for the title plant on phenolic composition. In Kukić (2006) [24] are reported the total phenolic data of four species, *S. anisochila*, *S. beckeana*, *S. plumosa* and *S. alpina* ssp. *Dinarica*, that result in a ranging concentrations 65-192 mg/g of dry extract. Total phenol content in different species (*S. setifera* C. A. Mey. *S. inflata*, *S. persica*, *S. byzantina*, *S. laxa*, *S. turcomanica*, *S. subaphylla* and *S. trinervis*) result in ranging concentration from 4.3 (in *S. trinervia*) to 44.5 (in *S. fruticulosa*) mg/g dried plant. [25].

### 3.2 Antioxidant activity

Ethanol extract of *S. glutinosa* were screened for antioxidant activity by six test systems: ABTS assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging,  $\beta$ -carotene/linoleic acid assay, scavenging of hydrogen peroxide, superoxide anion scavenging ( $\bullet\text{O}_2^-$ ) assay, and hypochlorous acid scavenging (HOCl) assay. Table 2 reported the antioxidant activity of ethanol extract of *S. glutinosa*. The chemical complexity of ethanolic extracts, often a mixture of dozens of compounds with differences in functional groups and chemical behaviour, could lead to scattered results, depending on the test employed. Therefore, an approach with multiple assays in screening work is highly advisable. Among different ways in use to report the antioxidant activity, the amount of sample necessary to reduce the oxidation by 50 % ( $\text{IC}_{50}$ ) was used. The in vitro antioxidant activities of the extract tested were compared to those of Trolox or BHT, widely used as reference drugs in literature.

ABTS and DPPH radical scavenging methods are common spectrophotometric procedures for determining antioxidant capacities of components [26]. DPPH radical involves a hydrogen atom transfer process, and ABTS radical involves an electron transfer process [27]. The antioxidant activity on DPPH and ABTS radicals of *S. glutinosa* may be attributed to a direct role in trapping free radicals by donating hydrogen atom or electron.

Dry extract was diluted in ethanol at 1 mg/ml, and the solution directly tested. As reference antioxidant a ranging concentration of trolox solutions were used, in order to determine a range for linear response. The  $\text{IC}_{50}$  activity of extract at 1mg/ml results  $2.4 \pm 4$  (ABTS assay) and  $2.0 \pm 3$  (DPPH assay) expressed as mg of Trolox equivalents. Compared with the reference, *S. glutinosa* is about twice time less active. Lipid peroxidation may be described as the oxidative deterioration of unsaturated fatty acids caused by free radicals and the changes resulting from this process.  $\beta$ carotene/linoleic acid assay were chosen as the test for the determination of ethanolic extract of *S. glutinosa* antioxidant activity on polyunsaturated fatty acids. The autooxidation of linoleic acid in the negative control group increased rapidly on day 1, and reached maximum level on day 4. In our study, *S. glutinosa* significantly inhibited the degree of lipid peroxidation. The activity may be due to termination of the radical chain reaction after its propagation by scavenging highly toxic lipid peroxy radical as the curve conc/lipid peroxide inhibition suggests. The  $\text{IC}_{50}$  activity of extract results  $12 \pm 3$  expressed as mg of BHT equivalents. Compared with the reference, *S. glutinosa* is less active, but owing the high specificity of BHT there was an high lipid peroxidation. The activity in lipid peroxidation test results higher than Trolox ( $0.1 \pm 0.02 \text{ mg}_s/\text{mg}_r$ ). We also investigated the ability of ethanol extract to scavenge in vitro oxygen-derived species, such as hydrogen superoxide anion ( $\bullet\text{O}_2^-$ ), peroxide ( $\text{H}_2\text{O}_2$ ), and hypochlorous acid (HOCl) (which are known to be released, among other mediators, by activated phagocytic cells during inflammatory disorders). Both ( $\bullet\text{O}_2^-$ ) and  $\text{H}_2\text{O}_2$ , like HOCl and OCl $\cdot$ , are powerful oxidants capable to react with amines, amino acids, sulphhydryls, disulfides, thioethers, aromatics and a variety of potentially important biological substrates. For this reason, the discovery of some compounds with excellent hydroxyl radical scavenging ability would be significant for some ailments induced by oxidative

stress. Superoxide anion is an oxidant that can generate more dangerous species, including singlet oxygen and hydroxyl radicals, also hydrogen peroxide may induce hydroxyl radicals, which would result in great damage to cells. Hydroxyl radicals are highly strong reactive oxygen species, and there is no specific enzyme to defense against them in human body [28-30]. Horseradish peroxidase (HRPO), S.A.S., and taurine assays were chosen for the determination of antioxidant activity on hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), (<sup>•</sup>O<sub>2</sub><sup>-</sup>) and hypochlorous acid (HOCl). The ethanolic extract revealed an antioxidative effects, showing IC<sub>50</sub> values in the range 0.17-0.22 mg: 0.22± 0.05 (HRPO assay), 0.24+0.06 (S.A.S. assay), and 0.17+0.04 (taurine assay) (Table 2). The extent of the effects can be compared to that of trolox, 1 mg trolox being equivalent to 1.1-3.2-2.5 respectively. In the test, *S. glutinosa* showed relevant scavenging activity on the three reactive oxygen species that can be, at least in part, justified by polyphenols presence in the extract.

### 3.3 Cytotoxic activities

In figures 1-3 were reported data of percentage inhibition of cells treated with different doses of extract. The extract from *S. glutinosa* was evaluated for *in vitro* cytotoxic activities on tumor (HepG2, human hepatocarcinoma and MCF7, breast adenocarcinoma) and non tumor (C2C12, mouse myoblast) cell lines, by MTT test. Cells were stimulated at two different doses of 100 and 200 µg/ml and cell viability quantified after 24 and 48h. In all experiments extract reduce cell viability in a dose dependent response. The most sensible cell lines results the HepG2, after 24h. The stimulation time influence weakly the cells viability, that result more reduced after 48h. On HepG2 the effect of longer time exposure results as an evident reduction of toxicity. In HepG2 and C2C12 the reduction in viability seems related to the dose of extract, while on MCF7, the higher dose of extract have only weak effect on cell proliferation. The extract showed stronger *in vitro* cytotoxicity on tumor cell lines, with higher proliferative inhibition at -37% and -27% respectively for HepG2 (24h) and MCF7 (48h) and result less effective against muscle cells C2C12 (cell viability -23%, after 48h). In experiments was detected an inhibition of cell proliferation always below 50%, so the resulting IC<sub>50</sub> is clearly higher than 200 mg/ml. Comparing to drugs used as reference (IC<sub>50</sub> of 5-fluorouracil: 15.9 µmol/l and 73-81 µM, respectively for HepG2 [31] and MCF7 [32]), the extract can be considered as a mild antiproliferative agent. More interesting for future perspective results the selective activity that seems reduce the viability only on tumor cell lines. No bibliographic data are available on antiproliferative activity of *S. glutinosa*. Only one report can be related to our experimental conditions. In Háznagy-Radnai, et al. (2008),[33] is reported that extracts from other species of *Stachys* (*S. recta*, *S. palustris*, *S. germanica*, *S. byzantina*), at 10 µg/ml on MCF7, afford a reduction of proliferation in 28-55% range. Compared to data of present work, the antiproliferative activity is similar, but the extract was tested concentration from 10 to 20 times lower. Experimental and bibliographic data confirm *S. glutinosa* as a weak inhibitor of tumor cell proliferation. Further studies will be dedicated to understand the effect of extract on cell metabolism, particularly in

antiradical and antioxidant mechanisms, that are clearly involved in pathogenesis of large number of degenerative diseases.

### 3.4 Antimicrobial activity

Extract obtained from flowers of *S. glutinosa* were assayed for their antimicrobial activity, by measuring the inhibition zone, in presence of different extract concentrations. Results given in Table 4, show that the ethanol extract exhibits an antibacterial activity against *E. coli*, *S. agaltilae* and *S. pyogenes*; Less sensible resulted *S. aureus* and *S. pneumoniae*, while no significant activity was detected against *P. aeruginosa*.

Further investigations are required to detect the relation between chemical composition and antimicrobial activity of the extract.

### 4. Conclusions

This research reports on the qualitative profile of *S. glutinosa* ethanolic extract, as well as previously reported in the literature (Serrilli et al. 2006; Karioti et al. 2010) The main phenylethanolic glycosides present were betonyoside, forsythoside and melittoside; caffeoylquinic acid derivatives and chlorogenic acid were identified as the predominant phenolic acids. The majority of the constituents were represented by isoscutellarein derivatives. This extract revealed antioxidative effects, showing IC<sub>50</sub> values in the range 0.17– 0.22 mg: 0.22 ± 0.05 (HRPO assay), 0.24 ± 0.06 (SAS assay) and 0.17 ± 0.04 (taurine assay). The biological assay of *S. glutinosa* extract showed significant scavenging activity on the three ROS that can be, at least in part, justified by the presence of polyphenols in the extract. The extract from *S. glutinosa* was also evaluated for in vitro cytotoxic activities on tumour (HepG2 and MCF7) and non-tumour (C2C12) cell lines, by MTT test. The extract showed stronger in vitro cytotoxicity on tumour cell lines, with higher proliferative inhibition at 237% and 227%, respectively, for HepG2 (24 h) and MCF7 (48 h). Experimental and bibliographic data confirm *S. glutinosa* as a weak inhibitor of tumour cell proliferation.

Table 1.

Chemical class components of *S. glutinosa*

Total phenols	Flavonoids	flavonols	Oligomeric proanthocyanidins
mg/g d.e. as gallic acid	mg/g d.e. as rutin	mg/g d.e. as (-)-epicatechin	mg/g d.e. as cyanidin chloridrate
93.56±3.62	15.21±2.05	6.7±1.20	21.02±1.78

Table 2. Antioxidant activity of *S. glutinosa* ethanolic extract

	IC <sub>50</sub> mg	±S.D.	mg <sub>s</sub> /mg <sub>r</sub> (mean value)
ABTS assay	0.32	0.06	2.4

DPPH assay	0.28	0.05	2.0
Linoleic assay	0.12	0.03	12
HRPO assay	0.22	0.05	1.1
S.A.S. assay	0.24	0.06	3.2
Taurine assay	0.17	0.04	2.5

A lower IC<sub>50</sub> value indicates greater antioxidant activity.

mg<sub>s</sub> = milligram of ethanolic extract with IC<sub>50</sub> activity ; mg<sub>r</sub> = milligram of Trolox or BHT with IC<sub>50</sub> activity

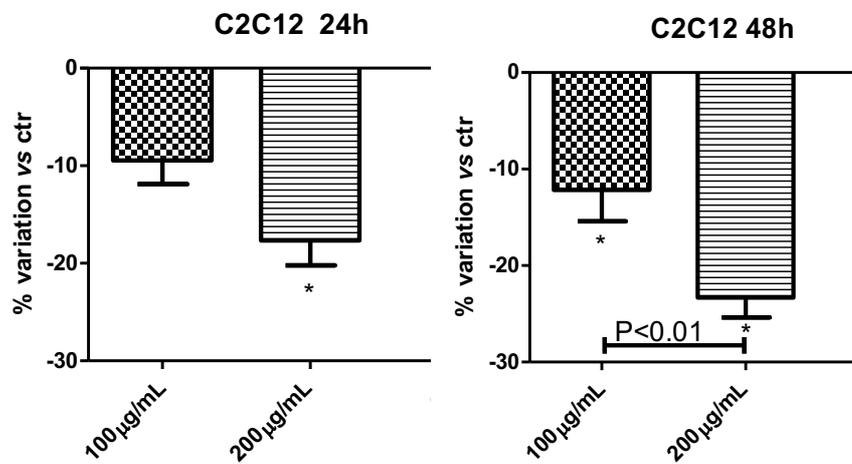
Table 3. Antimicrobial activity of ethanolic extract of *S. glutinosa*

Strains	inhibition zone (in mm) <sup>a</sup>		Chloramphenicol <sup>b</sup>
	Extract 1mg/ml	0.5 mg/ml	
<i>E. coli</i> (ATCC 13706)	12±3	7±2	31±5
<i>P. aeruginosa</i> (DSM 1117)	3±1	2±1	25±4
<i>S. agalactiae</i> (ISS1)	10±4	8±3	30±5
<i>S. aureus</i> (ATCC 25923)	9±3	6±2	35±6
<i>S. pneumoniae</i> (ISS3)	8±2	5±2	25±5
<i>S. pyogenes</i> (ISS2)	13±4	8±3	28±5

<sup>a</sup> Mean of three determinations ± SD.

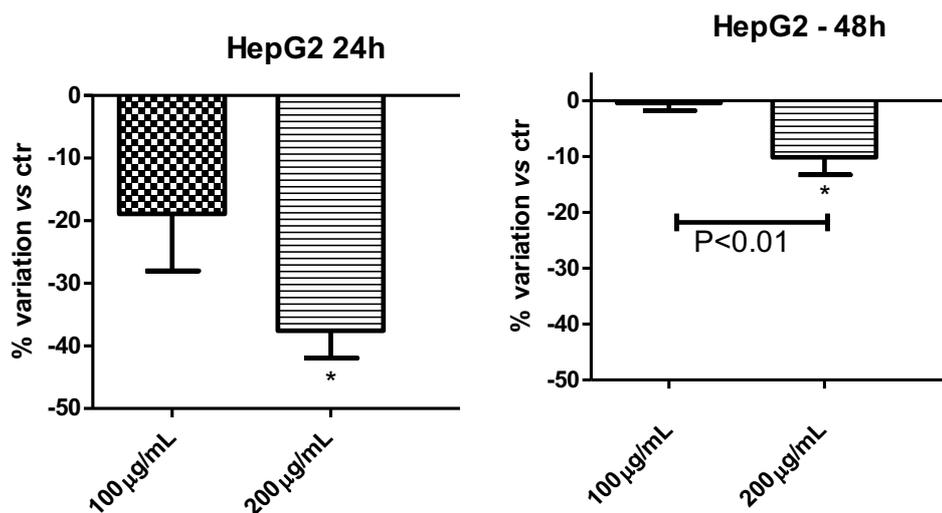
<sup>b</sup> Chloramphenicol (30 µg) was used as positive control for Gram-positive and Gram-negative bacteria; solvent control (DMSO) was negative for all tested strains.

**Fig. 1:** C2C12 cells viability 24 (a) and 48h (b) after stimulation with *S. glutinosa* extract.



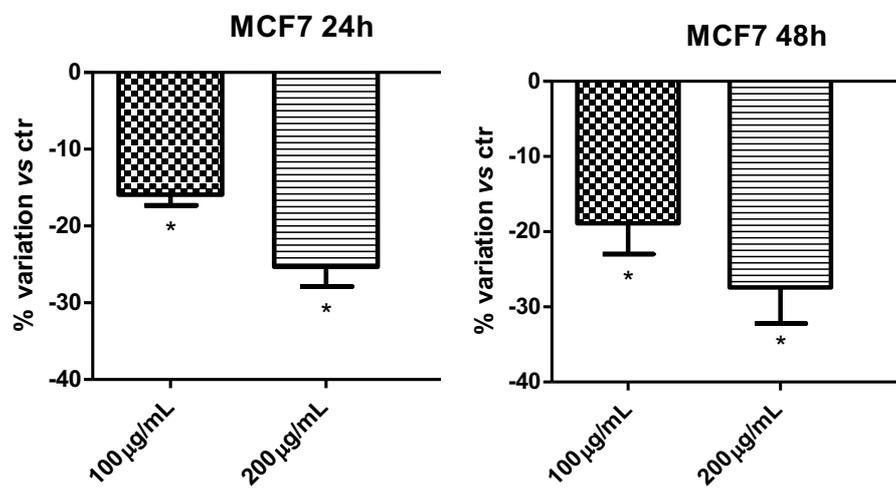
Data are expressed as mean % variation of cell viability vs control untreated group. ANOVA  $P < 0.01$ , \*sample vs control  $P < 0.01$

**Fig. 2:** HepG2 cells viability 24 (a) and 48h (b) after stimulation with *S. glutinosa* extract.



Data are expressed as mean % variation of cell viability vs control untreated group. ANOVA  $P < 0.05$ , \*sample vs control  $P < 0.05$

**Fig.3:** MCF7 cells viability 24 (a) and 48h (b) after stimulation with *S. glutinosa* extract.



Data are expressed as mean % variation of cell viability vs control untreated group. ANOVA  $P < 0.01$ , \*sample vs control  $P < 0.01$

## References

- [A] (Halliwell B. and Gutteridge J.M., *Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol*, 186: p. 1-85 1990.
- [B] (Halliwell B. and Gutteridge J.M.C., *Free radicals in biology and medicine. 2nd ed. Oxford: Clarendon Press. 543, 1989*)
- [C] (Pokorny W.L., *Natural antioxidants for food use. Trends Food Sci Technol Sept*, 60: 223-227, 1991)
- [D](Dessi M. A., Deiana M., Rosa A., Piredda M., Cottiglia F., Bonsignore L., Deidda D., Pompei R. and Corongiu F.P. Antioxidant activity of extracts from plants growing in Sardinia. *Phytotherapy Res.* 15, 511-518, 2001, )
- [E]Saxena, M., Saxena, J., Pradhan, A. 2012 Flavonoids and phenolic acids as antioxidants in plants and human health. *International Journal of Pharmaceutical Sciences Review and Research* 16 (2) , pp. 130-134
- [F]Sova, M., 2012, Antioxidant and antimicrobial activities of cinnamic acid derivatives, *Mini-Reviews in Medicinal Chemistry* 12 (8) , pp. 749-767
- [G](Gharavi N, Haggarty S and El-Kadi AO, *Chemoprotective and carcinogenic effects of tert-butylhydroquinone and its metabolites. Curr Drug Metabol* 8:1-7 (2007))
- [H] (Juan C Argoti, Sofia Salido, Pablo J Linares-Palomino, Bernardo Ram'rez, Braulio Insuasty and Joaquín Altarejos, *Antioxidant activity and free radical-scavenging capacity of a selection of wild-growing Colombian plants, J Sci Food Agric* 2011; 91: 2399-2406)
- [I] Giorgio, Pintore; Mario, Chessa; Paola, Manconi; Stefania, Zanetti; Antonella, Deriu; Bruno, Tirillini *Chemical composition and antimicrobial activities of essential oil of Stachys glutinosa L. from sardinia Natural Product Communications (2006), 1(12), 1133-1136.*
- [L] Menghini L., Genovese S., Epifano F., Tirillini B., Ferrante C., Leporini L., *Antiproliferative, protective and antioxidant effects of artichoke, dandelion, turmeric*

*and rosemary extracts and their formulation, International Journal of Immunopathology and Pharmacology, 23 (2):601-610, 2010*

- [1] Pignatti, S., Edagricole, Bologna, 1982.
- [2] Camarda, I., Le piante endemiche della Sardegna. 70. *Stachys glutinosa* L., Bollettino della Società Sarda di Scienze Naturali. 19 (1980) 261-7.
- [3] Atzei, A., Le piante nella tradizione popolare della Sardegna., Carlo Delfino Editore, 2003.
- [4] Mariotti, J.P., Costa, J., Bianchini, A., Bernardini, A.F., Casanova, J., Flavour and Fragrance Journal. 12 (1997) 205-9.
- [5] Pelissier, Y., Marion, C., Quastana, C., Milhau, M., Malan, A., Bessiere, J.M., Rivista Italiana EPPOS. 7 (1996) 526-35.
- [6] Mariotti, J.P., Tomi, F., Bernardini, A.F., Costa, J., Casanova, J., Rivista Italiana EPPOS. 7 (1996) 536-40.
- [7] Serrilli, A.M., Ramunno, A., Piccioni, F., Serafini, M., Ballero, M., Bianco, A., Monoterpenoids from *Stachys glutinosa* L., Nat. Prod. Res. . 20 (2006) 648-52.
- [8] Singleton, V.L., Rossi, J.A.J., Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, American Journal of Enology and Viticulture. 16 (1965) 144-53.
- [9] Lamaison, J.L., Carnat, A., Teneurs en principaux flavonoides des fleurs et des feuilles de *Crataegus monogyna* Jacq. et de *Crataegus laevigata* (Poiret) DC., Pharm Acta Helv. 65 (1990) 315-20.
- [10] Porter, L.J., Tannins, in: Dey, PM, Harborne, J.B. (Eds.) (Ed.), Methods in Plant Biochemistry, Academic Press, London, 1989, pp. 407.
- [11] Porter, L.J., Hrstich, L.N., Chan, B.G., The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin, Phytochemistry. 25 (1986) 223-30.
- [12] Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radical Biology and Medicine. 26 (1999) 1231-7.
- [13] Peterson, D.M., Hahn, M.J., Emmons, C.L., Oat avenanthramides exhibit antioxidant activities in vitro, Food Chem. 79 (2002).
- [14] Dapkevicius, A., Venskutonis, R., Van Beek, T.A., Linssen, P.H., . , Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania, J. Sci. Food Agric. 77 (1998) 140-6.
- [15] Gressier, B., Lebegue, S., Brunet, C., Luyckx, M., Dine, T., Cazin, M., et al., Pro-oxidant properties of methotrexate: evaluation and prevention by an anti-oxidant drug., Pharmazie. 49 (1994) 679-81.

- [16] Pick, E., Keisari, Y., A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture., *J. Immunol. Methods.* 38 (1980) 161-70.
- [17] Jung, I.L., Kim, I.G., Thiamine protects against paraquat-induced damage: scavenging activity of reactive oxygen species, *Environmental Toxicology and Pharmacology.* 15 (2003) 19-26.
- [18] Halliwell, B., Gutteridge, J.M.C., Oxygen is a toxic gas-an introduction to oxygen toxicity and reactive oxygen species, in: Halliwell, BaG, J.M.C. (Ed.), *Free Radical Biology and Medicin*, Oxford University Press, New York,, 1997, pp. 1-140.
- [19] Rakotoarison, D.A., Gressier, B., Trotin, F., Brunet, C., Dine, T., Luyckx, M., et al., Antioxidant activities of polyphenolic extracts from flowers, in vitro callus and cell suspension cultures of *Crataegus monogyna*, *Pharmazie.* 52 (1997) 60-4.
- [20] Weiss, S.J., Klein, R., Slivka, A., Wei, M.J., Chlorination of taurine by human neutrophils. , *J. Clin. Invest.* 70 (1982) 598-607.
- [21] Gressier, B., Cabanis, A., Lebegue, S., Brunet, C., Dine, T., Luyckx, M., et al., Decrease of hypochlorous acid and hydroxyl radical generated by stimulated human neutrophils: comparison in vitro of some thiol-containing drugs, *Meth. Find. Exp. Clin. Pharmacol.* 16 (1994) 9-13.
- [22] Mukhrizah, O., San Loh, H., Wiart, C., Jin Khoo, T., Hon Lim, K., Nee Ting, K., Optimal methods for evaluating antimicrobial activities from plant extracts,, *Journal of Microbiological Methods.* 84 (2011) 161-6.
- [23] Wayne, P.A., *Performance standards for antimicrobial susceptibility testing*, 2008.
- [24] Kukić, J., Petrović, S., Niketić, M., Antioxidant activity of four endemic *Stachys* taxa, *Biol Pharm Bull.* 29 (2006) 725-9.
- [25] Khanavi, M., Hajimahmoodi, M., Cheraghi-Niroomand, M., Kargar, Z., Ajani, Y., Hadjiakhoondi, A., et al., Comparison of the antioxidant activity and total phenolic contents in some *Stachys* species, *African Journal of Biotechnology.* 8 (2009) 1143-7.
- [26] Awika, J.M., Rooney, L.W., Wu, X.L., Prior, R.L., Cisneros-Zevallos, L., Screening methods to measure antioxidant activity of *Sorghum* (*Sorghum bicolor*) and *Sorghum* product., *Journal of Agricultural and Food Chemistry.* 51 (2003) 6657-62.
- [27] Kaviarasan, S., Naik, G.H., Gangabthagirathi, R., Anuradha, C.V., Priyadarsini, K.I., In vitro studies on antiradical and antioxidant activities of fenugreek (*Trigonella foenum graecum*) seeds, *Food Chemistry.* 103 (2007).
- [28] Halliwell, B., Reactive oxygen species in living systems: source, biochemistry, and role in human disease, *American Journal of Medicine.* 91 (1991).
- [29] Halliwell, B., Chirico, S., Lipid peroxidation: its mechanism, measurement, and significance, *American Journal of Clinical Nutrition.* 57 (1993) 715S-24S.

[30] Liu, C.Z., Yu, J.C., Zhang, X.Z., Wang, T., Han, J.X., On changes of activity of antioxidases in hippocampus of rats with multi-infarct dementia and the intervention effects of acupuncture, *China Journal of Traditional Chinese Medicine and Pharmacy*. 20 (2005) 724-6.

[31] He, L.Q., Liu, J., Yin, D.K., Zang, Y.H., Wang, X.S., Synthesis and biological evaluation of nitric oxide-releasing matrine derivatives as anticancer agents, *Chinese Chemical Letters*. 21 (2010) 381-4.

[32] Grosse, P.Y., Bressolle, F., Pinguet, F., In vitro modulation of doxorubicin and docetaxel antitumoral activity by methyl- $\beta$ -cyclodextrin. *European journal of cancer*. 34 (1998) 168 -74.

[33] Háznagy-Radnai, E., Réthy, B., Czigle, S., Zupkó, I., Wéber, E., Martinek, T., et al., Cytotoxic activities of *Stachys* species, *Fitoterapia*. 79 (2008) 595-7.